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Synthesis of New Fluorogenic Cyanine Dyes and Incorporation into RNA Fluoromodules

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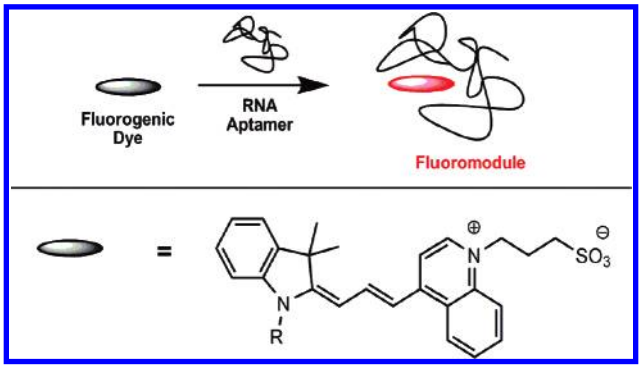
ABSTRACT



A new fluorogenic cyanine dye was synthesized and found to have low fluorescence quantum yield in fluid solution and in the presence of double-stranded DNA but 80-fold enhanced fluorescence in viscous glycerol solution. An RNA aptamer selected for binding to the new dye exhibits K_d = 87 nM and 60-fold fluorescence enhancement. The dye–aptamer pair is a fluoromodule that can be incorporated into fluorescent sensors and labels.

Fluorogenic cyanine dyes such as thiazole orange (TO, Figure 1), oxazole yellow (YO), and their derivatives are widely used for fluorescence imaging and detection of DNA and RNA.[1] These dyes have also found numerous applications as signaling elements in sensors, where they are covalently attached to recognition moieties such as nucleic acid oligomers[2] or peptides.[3] Enhanced fluorescence from the dye signals binding of the sensor to its target.

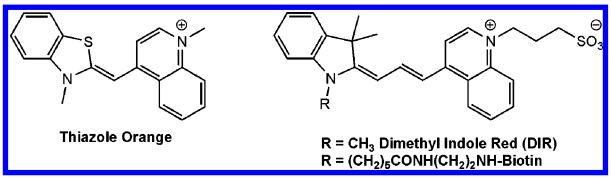


Figure 1. Fluorogenic cyanine dyes.

The fluorogenic behavior of unsymmetrical cyanines such as TO and YO arises from restriction of excited-state twisting about the central methine bridge separating the two heterocycles,[4] which normally leads to rapid nonradiative deactiva-

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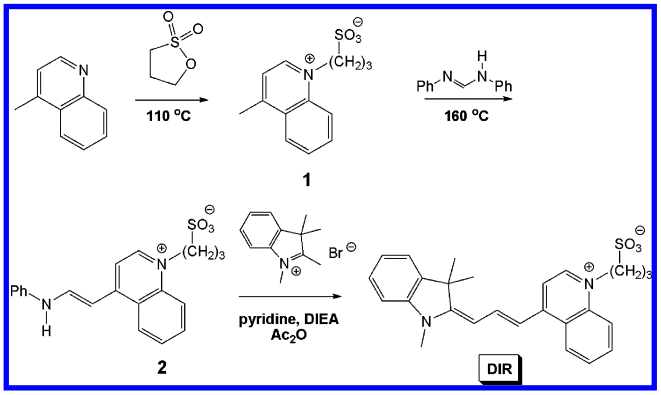
tion to the ground state.[5] Viscous solvents or restrictive local environments (such as a DNA intercalation site) inhibit this motion and lead to significantly enhanced (i.e., 10^1-10^3-fold) fluorescence quantum yields (ϕ_f).

While most applications for fluorogenic cyanines have been for in vitro analysis and detection, we are interested in developing a catalog of intracellular labels and sensors. These *fluoromodules* would consist of specific dye−RNA or dye−protein partners where the RNA or protein apomodule is genetically encoded and expressed, whereas the dye is delivered exogenously into the cell. The obvious complication in using an unsymmetrical cyanine as the dye component is the strong tendency of these dyes to bind nonspecifically to cellular DNA and RNA, which would compete with the specific RNA or protein apomodule. Herein we report the design and synthesis of a new fluorogenic cyanine dye with significantly reduced affinity for double-stranded DNA and nonspecific RNA. This dye was then used to select high affinity fluorescence-activating RNA aptamers from a combinatorial library.

The target dye, dimethylindole red (DIR), is shown in Figure 1. Two features were intended to suppress nonspecific DNA binding: the bulky dimethylindole heterocycle should hinder intercalation between π-stacked base pairs in DNA or RNA, while the anionic propylsulfonate substituent on the quinoline ring system introduces nonspecific electrostatic repulsion from polyanionic nucleic acids. While the same repulsion would reduce binding affinity for a specific RNA partner, the ability to select high affinity RNA aptamers for negatively charged small molecules such as GTP indicated that the electrostatic repulsion could be compensated by other specific binding interactions.[6]

The synthesis of DIR is shown in Scheme 1. Alkylation

Scheme 1. Synthesis of Dimethylindole Red (DIR)



of 4-methylquinoline with propanesultone yielded inner salt **1**, which was subsequently converted to the reactive hemidye **2** by condensation with *N*,*N*-diphenylformamidine. The dye was formed by reaction of **2** with 1-methyl-2,3,3-trimethyl-3*H*-indolium bromide under basic conditions.

DIR has intense absorbance in the red region of the visible spectrum with $\lambda_{max} = 602$ nm and $\epsilon_{max} = 134\,000$ M^{-1} cm^{-1} in methanol. The dye also exhibits the expected fluorogenic behavior: fluorescence is very low in aqueous buffer but increases ca. 80-fold in a solution of 90% glycerol in water (Figure 2). Meanwhile, in the presence of double-stranded

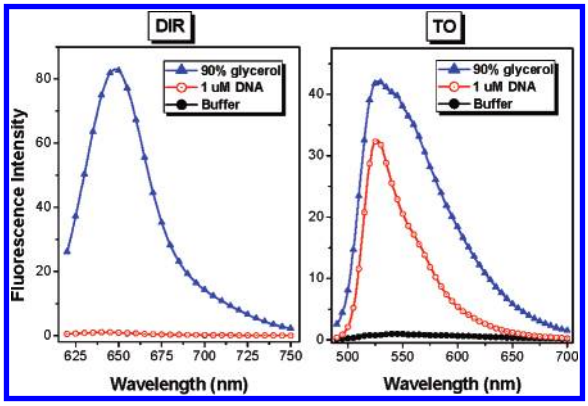


Figure 2. Fluorescence emission spectra recorded for DIR (left) and TO (right) in aqueous buffer, calf thymus DNA, and 90% glycerol. [Dye] = 1.0 μM, [DNA] = 100 μM base pairs. Buffer and DNA spectra overlap for DIR.

DNA, the fluorescence of DIR increases only 2-fold. For comparison, Figure 2 also shows the same experiments done with TO. The fluorescence for this dye is significantly enhanced both by glycerol and DNA, further verifying that the structural elements designed into DIR successfully suppress nonspecific binding to DNA.

We next used an affinity chromatography-based in vitro selection method to obtain RNA aptamers for DIR.[7] A biotin-conjugated analogue of DIR was synthesized as described in the Supporting Information and immobilized on a column packed with streptavidin−agarose beads. A naïve RNA pool containing $\sim 10^{14}$ unique sequences was obtained by in vitro transcription of the corresponding DNA pool. The pool was designed to contain a short internal self-complementary sequence that is expected to fold into a hairpin structure. A similar library was used by Davis and Szostak to obtain high-affinity aptamers for GTP.[6]

The RNA pool was subjected to multiple rounds of affinity selection and amplification by reverse-transcription PCR. The ability of the enriched pool obtained after each selection-amplification cycle to enhance the fluorescence of DIR was tested. Fluorescence enhancement first appeared in round 9, and selection was continued through round 15, at which point individual aptamers were isolated by cloning into *E. coli*.

A total of 32 RNA aptamers were screened for DIR fluorescence enhancement. As shown in Figure 3, six of these aptamers yielded greater than 5-fold enhancement, including three that enhance DIR fluorescence more than 20-fold. Thus, while the selection was based solely on affinity for DIR, a significant percentage of the aptamers sufficiently restrict

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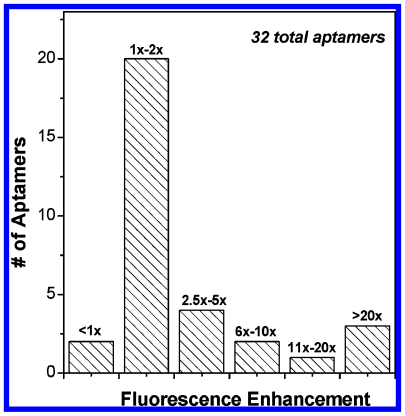

Figure 3. DIR fluorescence enhancement by RNA aptamers obtained from in vitro selection. [DIR] = [RNA] = 200 nM.

the conformational mobility of DIR to generate large fluorogenic effects.

One of the aptamers (DIR-Apt1) was chosen for further study. This aptamer strongly enhances DIR fluorescence, whereas the starting RNA pool has no effect on dye fluorescence (Figure 4). Two experiments were performed to

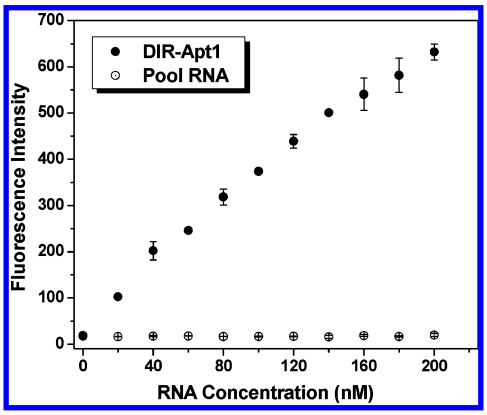

Figure 4. Fluorescence enhancement of DIR by DIR-Apt1 and naïve RNA pool. Samples contained 200 nM DIR and were excited at 602 nm.

verify the specificity of binding. First, the fluorescence enhancement of DIR-Apt1 was unaffected by a 10-fold excess of the starting RNA pool (data not shown). Second, addition of an RNA aptamer selected for binding to the structurally unrelated dye malachite green[8] enhances DIR fluorescence less than 2-fold (Figure S1, Supporting Information).

A continuous-variations experiment verified that DIR-Apt1 forms a 1:1 complex with DIR (Figure S2, Supporting Information). Fluorescence titration experiments were then used to determine the dissociation constant for the dye−aptamer complex: $K_D = 86 \pm 17$ nM (Figure 5). Thus, in

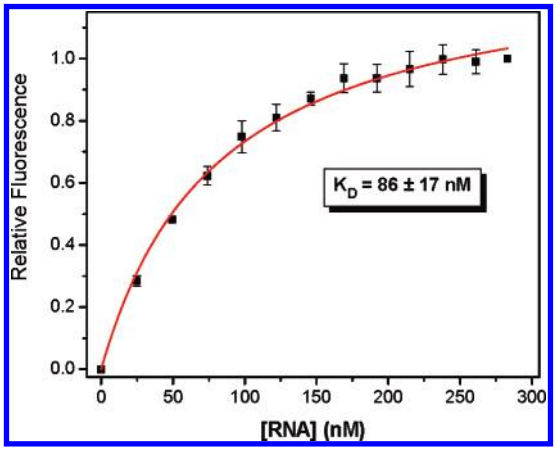

Figure 5. Fluorescence titration experiment for determining K_D of DIR−aptamer interaction. [DIR] = 100 nM. Data points and error bars represent mean and standard deviation from three separate trials. Red line is fit to 1:1 binding model.

spite of the anionic sulfonate group and the lack of strong hydrogen-bonding groups, a high-affinity RNA aptamer was readily selected for binding to DIR.

There are two other examples in the literature of RNA fluoromodules. In one case, an RNA aptamer for malachite green binds with $K_D = 117$ nM and enhances fluorescence more than 1000-fold.[9] While the fluorescence enhancement is greater than for the DIR fluoromodule reported here, the cyanine dyes are much better suited to obtaining a catalog of variable-color fluoromodules than are the triphenylmethyl dyes represented by malachite green. Meanwhile, Sparano and Koide selected RNA aptamers for a dimethylaniline (DMA) derivative.[10] Attachment of the DMA group to fluorescein led to substantial quenching of the dye fluorescence, most likely because of electron transfer from DMA to the dye. Addition of the DMA-binding RNA aptamer restored the fluorescence, although micromolar concentrations of the aptamer were required. This strategy is potentially generalizable to any dye that can be quenched by the DMA group, provided the necessary dye−DMA conjugate can be prepared.

Most recently, Sando and co-workers reported selection of DNA aptamers for derivatives of Hoechst 33258 that were engineered to have low binding to double-stranded DNA.[11] While the DNA aptamers cannot be expressed in cells, it is reasonable to expect that genetically encodable RNA aptamers could be selected for the same dye. The main advantage of our fluorogenic cyanines over Hoechst analogues is the broader wavelength range that is accessible to the cyanines.

The fluoromodule described here should find applications as a signaling component for sensors. For example, the DIR-Apt1 aptamer could be fused with another aptamer that binds

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to a separate target in order to create allosteric bifunctional aptamers that bind DIR and, therefore, "light up" only when the target molecule is bound.[12] Alternatively, genetic encoding of aptamers has been used to regulate translation of mRNA through reversible binding of a small molecule to its cognate aptamer that is transcribed along with the RNA of interest.[13] Similarly, the DIR-binding aptamer could be genetically encoded and expressed inside cells not only for controlling translation but alternatively for detecting transcription. In addition, DIR should be readily applied to selection of protein apomodules based on single-chain antibody fragments using the approach described recently by our center.[14]

In conclusion, we report the design and synthesis of a new fluorogenic dye, dimethylindole red, and selection of high-affinity RNA aptamers that activate the fluorescence of the dye. The fluorogenic behavior of the dye is likely due to torsional restriction of the excited state when in a viscous solvent or when confined by a tightly binding, specific RNA aptamer. While other fluorescence-activating aptamers have been reported, the novelty of our approach lies in the rational design of the fluorogenic dye. For years, unsymmetrical cyanines have been designed to bind to DNA through nonspecific intercalation. Incorporating sterically bulky and/or anionic substituents (as in DIR) yields dyes that retain fluorogenic behavior in response to conformational restriction while suppressing nonspecific binding to DNA and RNA. Using these strategies together with established variations in heterocycle structure and polymethine bridge length will produce a family of dyes spanning the visible and near-IR regions of the spectrum. Combining rational design and synthesis of these dyes with the power of in vitro selection should ultimately yield a catalog of dye−RNA fluoromodules for applications in fluorescence sensing and labeling.

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Supporting Information Available: Synthetic details and spectral data for DIR and DIR−biotin conjugate; competition experiments with malachite green (MG) and MG-binding aptamer; and continuous-variations experiment to determine binding stoichiometry for DIR and DIR-Apt1. This material is available free of charge via the Internet at http://pubs.acs.org.

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